

Exhibit B

Azithromycin Modulates Murine Immune Responses to Pneumococcal Conjugate Vaccine and Inhibits Nasal Clearance of Bacteria

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Macrolide antibiotics, including azithromycin, have been implicated in the modulation of host immune responses, independently of their antimicrobial properties. The present work was designed to study the effect that azithromycin has on protective humoral immune responses induced by a 7-valent, polysaccharide, pneumococcal conjugate vaccine (PCV7). By use of a murine vaccination/challenge model, it was found that inoculation with azithromycin led to significantly lower primary antibody responses, decreased recall proliferative responses, and, in nasal cavities, impaired clearance of *Streptococcus pneumoniae* serotype 14 from the nasal cavities. The results demonstrate that azithromycin can be inhibitory with regard to protective immune responsiveness.

Certain types of antibiotics have been implicated in the modulation of host immune responses, independently of their antimicrobial properties [1–3]. In particular, it has been shown that macrolide antibiotics affect various macrophage, monocyte, and lymphocyte functions, such as phagocyte chemotaxis, induction of the oxidative burst, and monocyte differentiation. These antibiotics can also modulate the migration of neutrophils, as well as the production of proinflammatory cytokines [2–7]; they might affect the host's immune response by inter-

fering with the activity of helper T cells [7]; and their anti-inflammatory properties may also be related to their ability to induce apoptosis of polymorphonuclear cells [8]. One previous study in mice has investigated the effect that certain antibiotics have on humoral immune responses [9].

The immunomodulatory effects of macrolides have also been noted in clinical settings [10, 11]. Recent clinical trials have shown that prolonged treatment with azithromycin has a beneficial effect on disease parameters in patients with cystic fibrosis; this effect did not appear to be directly related to this drug's antimicrobial properties [12, 13].

The major natural reservoir of *Streptococcus pneumoniae* is the human nasopharynx. Most pneumococcal invasive diseases are thought to be preceded by nasopharyngeal colonization [14]. Because (1) we wanted to study the impact that antibiotics may have on humoral immune responses to a pneumococcal conjugate vaccine and (2) humans can be vaccinated against pneumococcus when they are healthy as well as when they are receiving treatment for nonsevere bacterial illness, we also studied their effect on pneumococcal nasopharyngeal colonization.

We studied, in a mouse-model system, the effect that certain antibiotics (azithromycin, ceftriaxone, and ciprofloxacin, commonly used macrolide, β -lactam, and fluoroquinolone antibiotics, respectively) have on the acquired immune response to a 7-valent, polysaccharide, pneumococcal conjugate vaccine (PCV7). We also investigated whether bacterial clearance in vaccinated mice would be influenced by antibiotic treatment, because this effect could have major clinical implications.

Materials and methods. Female BALB/c mice, 6 weeks old, were used for the experiments. They were obtained from Charles River Laboratories through the National Cancer Institute and were housed at the Albany Medical College. All experimental protocols were approved by the Albany Medical College Animal Care and Use Protocol.

On day 0, a single 100- μ L dose of antibiotic was given intraperitoneally (ip) to each mouse 1 h prior to immunization. Each mouse in the experimental groups received either azithromycin (Pfizer) (prepared for injection use) at a dose of 50 mg/kg, ceftriaxone (Roche) at a dose of 100 mg/kg, or ciprofloxacin (Bayer) (prepared for injection use) at a dose of 50 mg/kg. Each mouse in the control group received 100 μ L of PBS. Neither the antimicrobial preparations nor the control preparations contained alcohols. The dosages used were designed to mimic, on a weight basis, the serum and tissue levels of these antibiotics achieved in humans undergoing treatment.

On day 0, each mouse was inoculated intramuscularly with

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PCV7 (Lederle Laboratories), which contains 7 different pneumococcal polysaccharides, including pneumococcal polysaccharide type 14 (PPS14) at 0.2 µg/dose. The vaccine was administered 1 h after antibiotic treatment.

On days -1 and 7, blood was obtained by retro-orbital bleeding of anesthetized mice. Blood was centrifuged at 2700 g at 5°C for 5 min. The recovered serum was aliquoted and stored at -70°C until antibody-titer measurements were performed.

Nunc-Immuno plates (Nalge Nunc International) were used for ELISA, to measure titers of specific antibody to PPS14. The plates were first coated with 50 µL of poly-L-lysine (100 mg/mL) and incubated at 4°C overnight. The following day, the plates were washed 3 times with PBS. Each well was incubated with 50 µL of PPS14 (15 µg/mL) at 4°C overnight. The following day, the plates were washed 3 times with 0.5% Brij (Sigma). The plates were then blocked with 5% fetal calf serum (Hyclone Laboratories) and 0.3% Brij in 10 mmol/L of PBS at room temperature for 1 h. Serial dilutions of serum in blocking buffer were added to the plates, which were then incubated at room temperature for 2 h. After 3 washes with 0.5% Brij, 50 µL of alkaline phosphatase-conjugated goat anti-mouse antibody against whole immunoglobulin—IgM, IgG1, or IgG2a (Southern Biotechnology)—were added, and the plates were incubated at room temperature for 1 h. After 3 washes with 0.5% Brij, *p*-nitrophenyl substrate (Sigma) was added, and, 30 min later, absorbance at 405 nm was measured with a microplate reader (Bio-Tek Instruments). Titers were determined by use of a 50%-maximal-binding end point.

One hour after ip injection of azithromycin, the mice were immunized ip with PCV7; 4 weeks after the priming, their spleens were removed, and cell suspensions were cultured in microtiter wells (2×10^5 cells/well). Splenocytes were stimulated with either different concentrations of PCV7—a T-independent B-cell mitogen—lipopolysaccharide (LPS) (10 µg/mL) from *Escherichia coli* (Sigma), or concanavalin A (ConA) (0.1 µg/mL) (Sigma)—a T-cell mitogen. The cultures were maintained at 37°C for 72 h, were pulsed with [³H]thymidine (ICN Radiochemicals) (1 µCi/well) for 18 h, and were harvested onto glass-fiber filters by use of a semiautomatic harvester (Tomtec Harvester96; Tomtec). [³H]thymidine incorporation was measured by use of a β-scintillation counter (1450 Microbeta Trilux; EG&G Wallac).

Pneumococcal strain TJ0983, which expresses PPS14 and is known to colonize mice rather than to cause systemic disease [14], was plated on blood-agar plates overnight and was cultured the following day at 37°C in Todd Hewitt broth supplemented with 0.5% yeast extract. The bacteria were identified on the basis of both colony morphology on blood-agar plates and sensitivity to optochin (Sigma). Bacteria were harvested by centrifugation and were washed twice in sterile PBS.

One week after antibiotic treatment and vaccination with PCV7, each anesthetized mouse was inoculated intranasally (inl)

with 1×10^5 cfu/mouse in 10 µL of Ringers solution. Three days after inoculation, the mice were euthanized, 100 µL of Ringers solution were injected into each mouse's trachea, and 50 µL of nasal secretions were collected from the tip of each nose. Serial dilutions were plated onto blood-agar plates containing gentamicin, with or without optochin, to assess pneumococcal growth.

For comparison of groups, the Mann-Whitney/Wilcoxon rank sum test was used. $P < .001$ was considered to be statistically significant.

Results. Macrolides can modulate the migration, oxidative burst, and differentiation of phagocytes, and they can also modulate cytokine production. To determine the effects that different types of antibiotics (azithromycin, ceftriaxone, and ciprofloxacin) have on murine primary humoral immune responses to PCV7, IgM, IgG1, IgG2a, and total serum antibody titers were measured 7 days after treatment. It was found that the total antibody titers in the group of vaccinated mice treated with azithromycin were significantly lower than those in the group of vaccinated mice treated with PBS (figure 1A). Similar results were obtained for IgG1 serum antibody titers (figure 1B), but not for IgG2a or IgM serum antibody titers. No statistically significant differences were found when the mean antibody titers of mice receiving either ceftriaxone (figure 1C) or ciprofloxacin (figure 1D) were compared with the mean antibody titers of mice in the control group.

To determine the potential effects that antibiotics have on the development of memory responses, recall-proliferation studies were next performed. Suspensions of splenocytes from vaccinated mice treated with azithromycin were found to have impaired memory responses to the PCV7 recall antigen when compared with those from the control groups treated with PBS (figure 2A). Memory responses to the mitogens LPS and ConA were not affected by treatment with azithromycin.

The effect that azithromycin has on nasopharyngeal carriage of *S. pneumoniae* serotype 14 was studied next. Seven days after an antibiotic and a vaccine were administered, mice were inoculated inl with *S. pneumoniae* serotype 14 (1×10^5 cfu/mouse). Three days later, nasal washes were performed to determine the extent of nasal colonization. The increase (in cfu/mL) in the group of mice that received azithromycin was statistically significantly higher than that in the control group treated with PBS (figure 2B). Thus, azithromycin inhibits the ability to clear nasal colonization by pneumococcus.

Discussion. Antibiotics continue to play a major role in the management of bacterial infections. Macrolide antibiotics, including azithromycin, are primarily used in the treatment of respiratory-tract infections. There is increasing evidence, however, that certain antibiotics, including macrolides, exert an immune modulatory effect on the host, independently of their antimicrobial effects. Because patients undergoing antibiotic

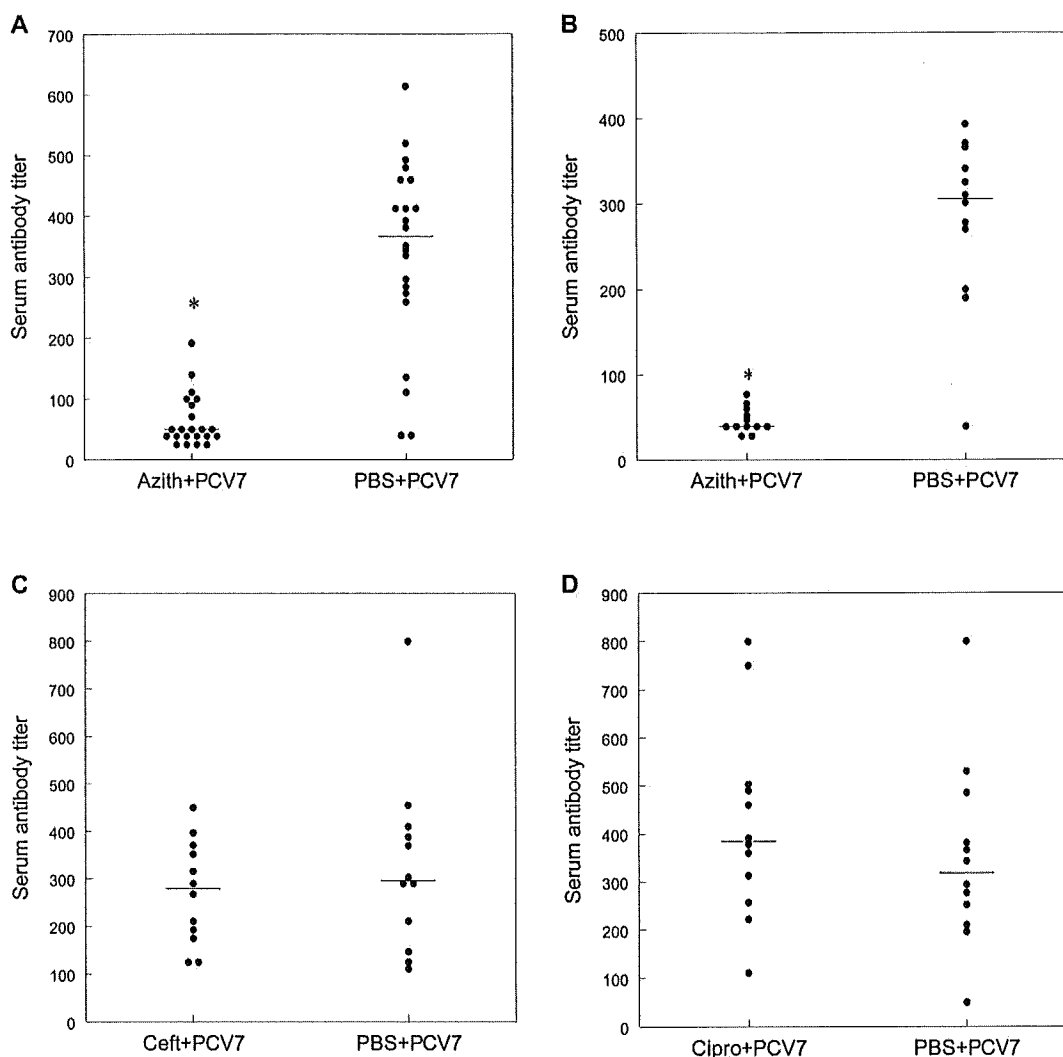


Figure 1. A, Decreased antibody titers in vaccinated mice that received azithromycin. Female BALB/c mice ($n = 22$ per group) were given either azithromycin intraperitoneally (ip) or PBS ip 1 h before intramuscular immunization with 100 μ L of 7-valent, polysaccharide, pneumococcal conjugate vaccine (PCV7) containing 0.2 μ g of pneumococcal polysaccharide type 14 (PPS14). Primary antibody responses to PPS14 were measured 7 days later by ELISA. The results in individual mice are indicated by the black circles, and the averages for the groups are indicated by the horizontal lines. The total antibody response was significantly lower in the azithromycin-treated group than in the PBS-treated control group (mean antibody titer, 63.09 ± 42.81 and 341.68 ± 152.7 , respectively; * $P < .001$, Mann-Whitney/Wilcoxon rank sum test). B, IgG1 subclass antibody titers in response to PCV7. Titers were significantly lower in the azithromycin-treated group ($n = 12$ mice) than in the PBS-treated group ($n = 12$ mice) (mean antibody titer, 46.08 ± 15.04 and 282.00 ± 99.16 , respectively; * $P < .001$, Mann-Whitney/Wilcoxon rank sum test). C and D, The total antibody response to PCV7 was not significantly affected by treatment with ceftriaxone (C) or ciprofloxacin (D) ($n = 12$ mice per group). Preimmune serum from each of the mice showed no binding to the plates. The data shown are the results of 3 experiments. Azith, azithromycin; Ceft, ceftriaxone; Cipro, ciprofloxacin.

treatment can receive vaccinations if the latter are clinically indicated, we studied the effect that certain antibiotics (azithromycin, ceftriaxone, and ciprofloxacin, commonly used macrolide, β -lactam, and fluoroquinolone antibiotics, respectively) has on the murine acquired immune response to PCV7.

Our results show that azithromycin modulates immune responses of mice immunized with PCV7. It dampens the primary humoral responses of mice to this vaccine as shown by a decrease in total antibody serum levels. This response is associated with decreased serum levels of IgG1 in the vaccinated group of mice

treated with azithromycin. In their study, Woo et al. found that, in response to a pneumococcal polysaccharide vaccine, a group of mice treated with clarithromycin had a decrease of total antibody titers, which was associated with a decrease of IgM titers but not with a decrease of IgG1 titers [9]; the pneumococcal polysaccharide vaccine that they used, however, is a T-cell-independent antigen, whereas we used a T-cell-dependent vaccine. The effect of azithromycin may be more profound on T-cell-dependent immune responses during the interaction between antigen-presenting cells, T cells, and B cells.

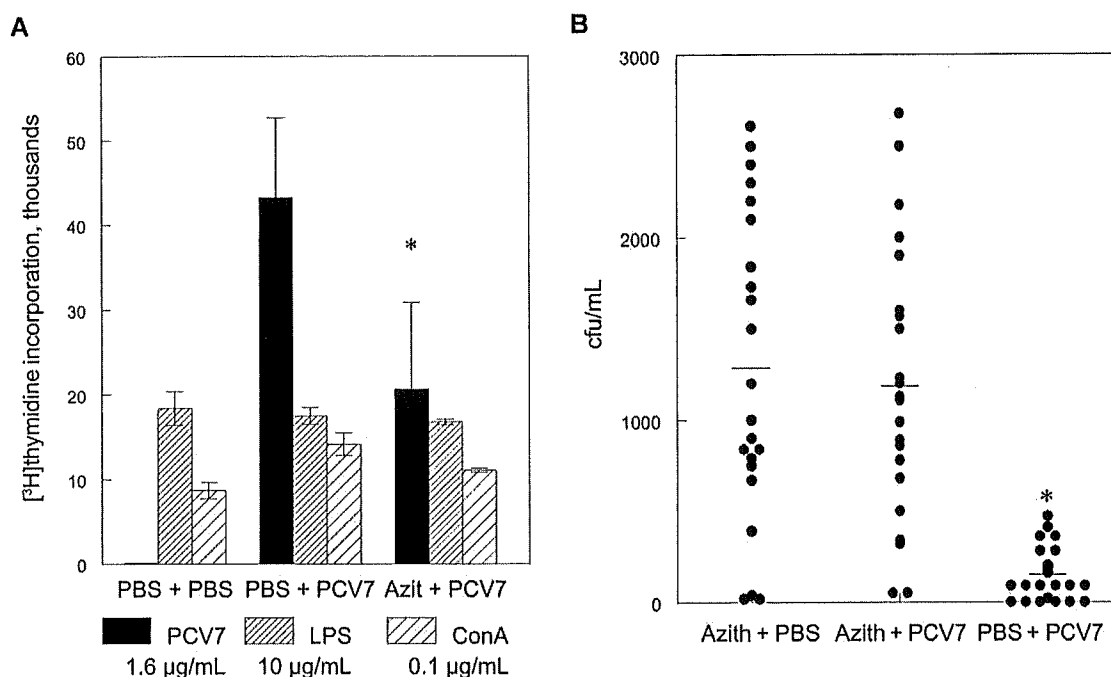


Figure 2. A, Azithromycin-treated mice, exhibiting depressed in-vitro recall responses to 7-valent, polysaccharide, pneumococcal conjugate vaccine (PCV7). Female BALB/c mice ($n = 16$ per group) were given either azithromycin or PBS intraperitoneally (ip) and given PCV7 ip 1 h later, as described in figure 1A. Four weeks later, their spleens were removed, and suspensions of splenocytes were cultured in microtiter plates (2×10^5 cells/well). Splenocytes were stimulated with PCV7, lipopolysaccharide, or concanavalin A. $[^3\text{H}]$ thymidine incorporation was measured after 72 h. The memory responses to the recall antigen in suspensions of splenocytes were significantly impaired in the azithromycin-treated mice than in those of the PBS-treated control group ($20,641 \pm 10,294$ and $43,306 \pm 9419$, respectively; $* P < .001$, Mann-Whitney/Wilcoxon rank sum test); the background response has been subtracted. Each well was run in triplicate. The data shown are the results of 3 experiments. Whiskers designate the SD. B, Impairment of nasal clearance of *Streptococcus pneumoniae* serotype 14 by azithromycin. Female BALB/c mice ($n = 22$ per group) were given either 1 mg azithromycin ip or 1 mg PBS ip 1 h before intramuscular immunization with $100 \mu\text{L}$ of PCV7 containing $0.2 \mu\text{g}$ of pneumococcal polysaccharide type 14. A third group of mice also received azithromycin followed by a PBS injection, but they did not receive PCV7. One week later, each mouse was inoculated intranasally with 1×10^5 cfu of *S. pneumoniae* serotype 14. Results of nasal washes were obtained and were plated for bacterial growth overnight. The results from individual mice are indicated by the black circles, and the averages for the groups are indicated by the horizontal lines. The number of cfu/mL were significantly higher in the azithromycin-treated mice than in the PBS-treated group (1184.54 ± 746.99 and 148.64 ± 149.55 , respectively; $* P < .001$, Mann-Whitney/Wilcoxon rank sum test). The data shown are the results of 3 experiments. Azith, azithromycin; Ceft, ceftriaxone; Cipro, ciprofloxacin.

Some investigators have reported the immunomodulatory effects that fluoroquinolones have on cytokine expression [1]; in our model, however, ciprofloxacin did not affect primary humoral responses in mice vaccinated with PCV7. Our results are more in accordance with the results of Jayakumar et al.'s studies done in rabbits, which show that ciprofloxacin does not affect specific immune responses [15]; in our experiment, the effect of ciprofloxacin and ceftriaxone was studied in only a small number of mice per group, and, therefore, small differences could not be excluded.

To determine the potential effects that azithromycin have on the development of memory responses, recall-proliferation experiments were performed. These experiments showed that azithromycin-treated mice exhibited depressed in-vitro recall responses to PCV7. Recall responses to the mitogens LPS and ConA were not affected by antibiotic treatment, which dem-

onstrates the antigen specificity of the effect. Surprisingly, the recall response to PCV7 was significantly greater than that to mitogens. The cause of this result is unknown but may be related to the alum used as an adjuvant in the vaccine.

Because nasal colonization with *S. pneumoniae* is thought to be important in the development of disease caused by this bacterium [14], the effect that azithromycin has on nasopharyngeal carriage of *S. pneumoniae* serotype 14 was studied. Our experiments showed that azithromycin inhibited the ability of infected mice to clear nasal colonization by pneumococcus. The reason for this is likely related to the inhibitory effect that azithromycin has on humoral responses and the possible impairment of opsonization in, and subsequent clearance of bacteria from, the respiratory tract.

Understanding the interplay between infection, immunity, and inflammation is critical for the improvement of vaccina-

tion protocols and prophylactic therapies against infectious diseases. Immune-based therapies are primarily viewed as being distinct, in their mode of action, from antimicrobial therapies. Although, because of their highly selective and specific action upon microorganisms, antimicrobial agents are generally considered to be safe and effective, observations made during the past decade have suggested that certain agents, in addition to their effect on bacterial growth, may influence host immune responses. The potential for antibiotics to influence the production of cytokines as well as vaccine-induced humoral immune responses, which has been suggested by other studies [1, 3–9, 15], could be of great importance—for example, when a patient is being treated with azithromycin and is in need of a pneumococcal vaccine. Perhaps a delay in immunization is warranted. More studies are needed to further characterize the cellular and molecular bases of these findings and their implications for clinical treatment.

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References

1. Purswani MU, Eckert SJ, Arora HK, Noel GJ. Effect of ciprofloxacin on lethal and sublethal challenge with endotoxin and on early cytokine responses in a murine in vivo model. *J Antimicrob Chemother* 2002;50: 51–8.
2. Hand WL, Hand DL, King-Thompson NL. Antibiotic inhibition of the respiratory burst response in human polymorphonuclear leukocytes. *Antimicrob Agents Chemother* 1990;34:863–70.
3. Takeshita K, Yamagishi I, Harada M, Otomo S, Nakagawa T, Mizushima I. Immunological and anti-inflammatory effects of clarithromycin: inhibition of interleukin 1 production of murine peritoneal macrophages. *Drugs Exp Clin Res* 1989;15:527–33.
4. Ianaro A, Ialenti A, Maffia P, et al. Anti-inflammatory activity of macrolide antibiotics. *J Pharmacol Exp Ther* 2000;292:156–63.
5. Kurdowska A, Noble JM, Griffith DE. The effect of azithromycin and clarithromycin on ex vivo interleukin-8 (IL-8) release from whole blood and IL-8 production by human alveolar macrophages. *J Antimicrob Chemother* 2001;47:867–70.
6. Culic O, Erakovic V, Cepelak I, et al. Azithromycin modulates neutrophil function and circulating inflammatory mediators in healthy human subjects. *Eur J Pharmacol* 2002;450:277–89.
7. Morikawa K, Zhang J, Nonaka M, Morikawa S. Modulatory effect of macrolide antibiotics on the Th1- and Th2-type cytokine production. *Int J Antimicrob Agents* 2002;19:53–9.
8. Koch CC, Esteban DJ, Chin AC, et al. Apoptosis, oxidative metabolism and interleukin-8 production in human neutrophils exposed to azithromycin: effects of *Streptococcus pneumoniae*. *J Antimicrob Chemother* 2000;46:19–26.
9. Woo PC, Tsoi H, Wong L, Leung HC, Yuen K. Antibiotics modulate vaccine-induced humoral immune response. *Clin Diagn Lab Immunol* 1999;6:832–7.
10. Van Vlem B, Vandholder R, De Paepe P, Vogelaers D, Ringoir S. Immunomodulating effects of antibiotics: literature review. *Infection* 1996;24:275–91.
11. Labro MT. Anti-inflammatory activity of macrolides: a new therapeutic potential? *J Antimicrob Chemother* 1998;41:37–46.
12. Wolter J, Seeney S, Bell S, Bowler S, Masel P, McCormack J. Effect of long term treatment with azithromycin on disease parameters in cystic fibrosis: a randomised trial. *Thorax* 2002;57:212–6.
13. Saiman L, Marshall B, Mayer-Hamblett N, et al. Azithromycin in patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa*: a randomized controlled trial. *JAMA* 2003;290:1749–56.
14. Wu H, Virolainen A, Mathews B, King J, Russell MW, Briles DE. Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice *Microb Pathog* 1997;23:127–37.
15. Jayakumar K, Honnagowda, Krishnappa G, Sastry KN, Narayana K. Effect of ciprofloxacin on specific immune responses in rabbits. *Indian J Exp Biol* 2002;40:111–4.